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Published in:
FEMS Microbiology Ecology

DOI:
10.1093/femsec/fiv079

Publication date:
2015

Citation for published version (APA):
Martínez-Fernández, G., Abecia, L., Martín-García, A. I., Ramos-Morales, E., Denman, S. E., Newbold, C. J., Molina-Alcaide, E., & Yáñez-Ruiz, D. R. (2015). Response of the rumen archaeal and bacterial populations to anti-methanogenic organosulphur compounds in continuous-culture fermenters. *FEMS Microbiology Ecology*, *91*(8), [fiv079]. https://doi.org/10.1093/femsec/fiv079

FEMS Microbiology Ecology Advance Access published July 15, 2015

Response of the rumen archaeal and bacterial populations to anti-methanogenic organosulphur compounds in continuous-culture fermenters

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Running Head: Anti-methanogenic compounds and rumen ecosystem

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Abstract

The study of methanogenesis inhibitors effectiveness in the rumen have shown inconsistent results, mainly due to poorly understood effects on the key microbial groups involved in methane CH_4 synthesis pathways. This experiment was designed to assess the effect of propyl propane thiosulfinate (PTS), diallyl disulfide (DDS) and bromochloromethane (BCM) on rumen fermentation, methane production and microbial populations on continuous culture fermenters. No effects on total VFA were observed with PTS and DDS, while were decreased with BCM. Amylase activity increased with BCM compared to the other treatments. A decrease on methane production was observed with PTS (48%) and BCM (94%) compared to control. Concentration of methanogenic archaea decreased with BCM from day 4 onward and with PTS on days 4 and 8. The pyrosequencing analysis revealed that PTS and BCM decreased the relative abundance of Methanomicrobiales and increased that of *Methanobrevibacter* and *Methanosphaera*. The concentration of total bacteria was not modified by any treatment, although BCM treatment increased the relative abundance of *Prevotella* and decreased that of *Ruminococcus*. These results suggest that the inhibition of methane production in the rumen by PTS and BCM is associated with a shift in the archaeal biodiversity and changes in bacterial community with BCM.

Keywords: Garlic compounds; methanogens; microbial community; rumen.

Introduction

Enteric microbial fermentation in ruminants is an important source of anthropogenic methane (CH_4), a potent greenhouse gas and its production represents a loss of around 2-12% energy for the animal, and consequently, a decrease of productivity (Morgavi *et al.*, 2010). The microbial populations responsible for fermentation in the rumen are comprised of an extremely diverse and complex mix of bacteria, protozoa, fungi and archaea. This enables the animal to digest and metabolize plant structural carbohydrates that otherwise could not be achieved with only its digestive enzymes. Methanogenic archaea fill the role in the rumen of terminal reducers of carbon, producing CH_4 mainly from H_2 and CO_2.

In the last decade, a wide range of compounds has been tested for their ability to reduce methane emissions (Benchaar & Greathead, 2011). Some plant secondary metabolites have shown promise due to their antimicrobial activity, including garlic-derived compounds (Hart *et al.*, 2008). However, inconsistent results together with adverse effects on fiber digestion and fermentation through inhibition of some bacterial groups have also been reported, with the magnitude of these adverse effects varying depending upon the types and doses and diet composition. Part of the inconsistency in the effects has been associated with the variety of compounds included in the plant extracts, which highlights the importance of using pure active compounds to understand the effects. In some cases, the effect is reversed after a few days of treatment (Soliva *et al.*, 2011). Thus, the research aiming to decrease CH_4 emissions from ruminants has to be built upon a correct understanding on the mechanisms of action involved in relation to the main microbial groups likely to be affected (bacteria and archaea).

We have recently observed that two organosulphur compounds, propyl propane thiosulfinate (PTS) and dialyl disulphide (DDS), strongly inhibit methane production (up to 96 and 62 %, respectively) in batch culture after 24 h incubation of goats' rumen fluid (Martínez-Fernández *et al.*, 2013). However, the persistency of such effect needs to be confirmed and

the microbial groups involved identified. This would enable an understanding into the mechanisms of action and facilitate practical implementation as a feeding strategy. Therefore, the aim of this work was to evaluate the effects of DDS and PTS on rumen fermentation, microbial abundances and community structure and on methane production in continuous-culture fermenters (CCF) inoculated with goats' rumen fluid.

Materials and methods

Fermenters, treatments, diet and animals

Eight CCF were used following the model of Muetzel *et al*. (2009) with an effective volume of 1000 mL. The treatments were control (without additive), DDS (purity of 80%), PTS (purity of 75%) and BCM that was included as antimethanogenic reference compound (positive inhibition control). BCM was entrapped in an α-cyclodextrin matrix (May et al., 1995) before being included in the diet to ensure its stability. The formulation was prepared in our laboratory as dry white powder in 1 to 2 kg batches and contained 10% to 12% (wt/wt) of BCM. The additives and doses were selected from previous results obtained in batch cultures (Martínez-Fernández *et al*., 2013): 80 μL L^{-1} per day for DDS, 200 μL L^{-1} per day for PTS and 160 mg L^{-1} per day for BCM. The DDS and BCM were provided by Sigma-Aldrich Chemical (catalog numbers 317691 and 48067, respectively); PTS was provided by DMC Research Center SL (Granada, Spain). The experimental diet (Table 1) was composed of alfalfa hay and concentrate in a 50:50 ratio.

Eight Murciano-granadina goats fitted with permanent rumen cannula were used as donors of rumen content for the experiment. Goats were adapted for 21 days to the experimental diet and were fed once a day (9:00 h) with free access to water and mineral salt block (Pacsa Sanders, Sevilla, Spain). Animals were cared by trained personnel in accordance with the Spanish guidelines for animal protection (Royal decree, 2005) and the European

Convention for the Protection of Vertebrates used for Experimental and other Scientific Purposes (European Directive, 2007). All the experimental procedures involved in this study were approved (Proc. CB-INAN 2012001) by the Animal Welfare Committee at the Institute of Animal Nutrition (CSIC, Spain).

Experimental procedure and sampling

Eight CCF (1L), as described by Muetzel *et al.* (2009), were used in two replicated incubation runs of 12 days each. Eight adult Murciano-Granadina goats fitted with ruminal canula were used as donors of ruminal contents. For each incubation run, two groups of four fermenters were inoculated (700 mL per fermenter) with a different pool, each one obtained from three different animals selected randomly. This resulted in four different pools as follows: pool 1 (goats 1, 2 and 3) and pool 2 (goats 4, 5 and 6), used in the first run; pool 3 (goats 2, 5 and 7) and pool 4 (goats 3, 6 and 8), used in the second run. Treatments (control, DDS, PTS and BCM) were randomly supplied to one of the 4 fermenters inoculated with each rumen content pool, receiving each pool all the treatments. Each fermenter was fed 16 g of fresh matter per day of the basal diet ground at 1 mm, in two equal portions at 09:00 and 14:00 h. Flow through fermenters was maintained by continuous infusion of artificial saliva (Muetzel *et al.*, 2009) at a rate of 40 mL h^{-1} and CO_2 was continuously infused to keep anaerobic conditions. Fermenters were maintained in a water bath at 39ºC. On days 0, 4, 8 and 12 of incubation 10 mL of the fermenters content were collected before the morning feeding for VFA analysis and DNA extraction and kept at -20ºC and -80 ºC, respectively. On days 4 and 12 of incubation 1 mL of the fermenters content was collected before feeding and frozen at -80ºC for determination of amylase and xylanase activities.

On day 12 of each incubation run in CCF a batch culture trial (Theodorou *et al.*, 1994), was carried out to incubate fermenters content for 24 h to measure CH_4 production. The content

of each fermenter was filtered through two layers of cheesecloth while bubbling with CO_2. The substrate incubated consisted of 500 mg of the diet fed to fermenters in 120 mL serum bottles with 60 mL of the fermenter content. Three replicates and a blank of each fermenter and treatment were used. Bottles were sealed with rubber stoppers and aluminum caps and incubated at 39ºC in a water bath. At 24 h after inoculation, the total gas volume was measured in each bottle and a sample of the gas was collected in a graduated syringe and transferred to a 5 mL vacuum tube (Venoject, Terumo Europe N.V., Leuven, Belgium) and then kept at room temperature before methane concentration was measured by gas chromatography (GC).

Chemical analysis and calculations

Dry matter (DM, method ID 934.01), ash (method ID 942.05), ether extract (method ID 7.045) and crude protein (CP, method ID 984.13) in samples were determined by the procedures of the Association of Official Analytical Chemists (AOAC, 2005). Gross Energy (GE) was measured with an adiabatic calorimeter (Model 1356, Parr Instrument Co., Moline, IL). The neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) contents were analysed following the methodology described by Van Soest *et al.* (1991) using an ANKOM Model 220 Fiber Analyzer (Macedon, NY). The α-amylase enzyme was used for NDF analysis in the concentrate, and both NDF and ADF were expressed exclusive of residual ash. The ADL content was determined by solubilization of cellulose in the ADF residue with 72% sulphuric acid.

To measure the enzymatic activities in fermenters content, cells were lysed using a Mini-Beadbeater (BioSpec Products, Inc., Bartlesville, OK, USA) for one minute to release intracellular enzymes, during all the process the samples were kept in ice. Cell material was removed by centrifugation (10,000×g, 10 min, 4 ºC) and the supernatant was used for

144 analyses. Xylanase (EC 3.2.1.8.) and amylase (EC 3.2.1.1.) activities were determined
145 (Giraldo *et al*., 2008) using oat beachwood xylan and soluble starch, respectively, as
146 substrates. Enzymatic activities were expressed as micromoles of glucose or xylose released
147 in 1 min from the corresponding substrates per mL of sample at 39 ºC and pH 6.5.
148 The individual VFA concentrations were analysed using the gas chromatography technique
149 described by Isac *et al.* (1994).
150 The CH_4 concentration was determined by GC using a HP Hewlett 5890, Packard Series II
151 gas chromatograph (Waldbronn, Germany). A sample of 0.5 mL of gas was injected using a 1
152 mL Sample-Lock® syringe (Hamilton, Nevada, USA).
153 The amount of methane produced (micromoles) was calculated by multiplying the total gas
154 produced (micromoles) with the concentration of methane obtained.
155
156 **Real-Time PCR Analysis**
157 Samples from the fermenters content were collected on days 0, 4, 8 and 12 for DNA
158 isolation. Samples were freeze-dried and thoroughly mixed by physical disruption using a
159 bead beater (Mini-bead beater 8, BioSpec Products, Bartlesville, USA) for 1 min before using
160 QIAGEN QIAamp® DNA stool mini kit (Qiagen Ltd., UK) following the manufacturer's
161 instructions but with higher temperature (95ºC) for lysis incubation. The DNA samples were
162 used as templates for quantify the copy numbers of 16S rRNA (for bacteria), methyl
163 coenzyme M reductase A (mcrA) gene (for methanogenic archaea), and 18S rRNA (for
164 protozoa) by real-time quantitative PCR (qPCR).
165 The yield and purity of the extracted DNA were assessed using NanoDrop ND-1000
166 Spectrophotometer (NanoDrop Technologies, Wilmington, DE).
167 Primer sets used were as follows: forward: 5´-GTG-STGCAYGGYTGTCGTCA-3´and
168 reverse: 5´-ACGT- CRTCCMCACCTTCCTC-3´ for total bacteria (Maeda *et al*., 2003) and

169 forward: 5´-GCTTTCGWTGGTAGT-GTATT-3´ and reverse: 5´-CTTGCCCTCYAATCGT-
170 WCT-3´for protozoa (Sylvester *et al*., 2004). The primer sets for detection and enumeration
171 of methanogenic archaea (mcrA) were forward: 5´-TTCGGTGGATCD-CARAGRGC-3´ and
172 reverse: 5´-GBARGTCGWAWC- CGTAGAATCC-3´ (Denman *et al*., 2007). Three
173 replicates of each extract were used and a negative control was loaded on each plate run to
174 screen for possible contamination or dimer formation and to set the back- ground
175 fluorescence for plate normalization.

176 Real-time PCR analyses were performed on iQ5 multicolor Real-Time PCR Detection
177 System (BioRad Laboratories Inc., Hercules, CA). One microliter of DNA extract was added
178 to amplification reactions (25 μL) containing 0.2 μL of each primer (10 μM) and 12.5 μL of
179 iQ SYBR Green Supermix (BioRad Laborato- ries Inc.). Cycling conditions were 95°C for 5
180 min; 40 cycles of 95°C for 15 s, 60°C for 30s, and 72°C for 55 s; and 72°C for 1 min. The
181 threshold cycle (amplification cycle in which product formation exceeds background
182 fluorescence) of each sample was determined during the exponential phase of amplification.
183 The absolute amount for each microbial group, expressed as the number of DNA copies/g of
184 fresh matter, was determined using standards. The qPCR standards consisted of the plasmid
185 pCR 4-TOPO (Invitrogen, Carlsbad, CA) with an inserted 16S, mcrA, or 18S gene fragment
186 corresponding to a conserved sequence of total bacteria, methanogenic archaea, or protozoa,
187 respectively. The number of gene copies present in the plasmid extracts was calculated using
188 the plasmid DNA concentration and the molecular mass of the vector with the insert. The
189 concentrated plasmid was serially diluted (10-fold) to generate a standard curve.
190

191 **Pyrosequencing and sequence analysis**

192 The yield and purity of the extracted DNA from day 12 before feeding, were assessed using
193 NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, USA). For

sequencing of the 16S rDNA gene using the Roche /454 system, previously published primers were modified to include the 454 adaptor sequences and sample specific barcodes allowing samples to be multiplexed. Amplification of the bacterial V1-V2 regions of 16S rRNA was performed using the primer pair 27F and 357R (Liu *et al.*, 2007) . The archaeal hypervariable V6 region of the 16S rRNA gene was amplified using the primer pair 958F and 1048 Rmajor (Galand *et al*., 2009). The PCR was performed in triplicate, in a total volume of 25 µL containing 10x PCR buffer, 10 mM dNTP mix, 10 µM of forward and reverse primers, 1U FastStart Polymerase, and 1 µL of DNA template. The amplification conditions were: an initial denaturation step at 95ºC for 2 min; 30 cycles of denaturation at 95ºC for 30s, annealing at 55ºC for 30s, and elongation at 72ºC for 2 min; and a final extension step at 72ºC for 7 min. The size of the PCR products was then checked on a 1% agarose gel electrophoresis. Then triplicates were pooled together and products were then purified using the short fragment removal method described by Roche using their GS FLX amplicon DNA preparation guide and AMPure beads. The purified PCR products were quantified using Quant-iT PicoGreen dsDNA quantification kit (Invitrogen) and mixed in equimolar amounts to 10^7 molecules μL^{-1} sample. The amplicon pooled libraries were pyrosequenced on a Roche 454 FLX Titanium. The flowgram (sff) files were converted to fasta DNA (fna) and quality score (qual) file on the 454 cluster and transferred onto a Linux based workstation running the Quantitative Insights Into Microbial Ecology (QIIME) software (Caporaso *et al*., 2010b). Sequences were filtered to exclude those with mismatches in the primer sequence, exceeding 6 homopolymer base runs or sequences containing ambiguous bases. The libraries were split according to the 10nt barcode incorporated into the forward primer. The error-corrections of amplicon pyrosequences were made using Acacia (Bragg *et al*., 2012). The OTUs were generated by clustering at 97% sequence identity using UCLUST (Edgar, 2010) for bacteria and CD-hit for archaea (Li & Godzik, 2006). Representative sequences were aligned to the

reads of the GreenGenes database (DeSantis Jr *et al.*, 2006) using PyNAST(Caporaso *et al.*, 2010a). Taxonomic classification was assigned using the Basic Local Alignment Search Tool (BLAST). Beta diversity was used to create principal coordinate analysis (PCoA) plots using weighted UniFrac distances. Unifrac (Lozupone & Knight, 2005).

Statistical analysis

Fermentation parameters and microbial population were analysed as a repeated measures univariate analysis using GLM procedure of SPSS (IBM SPSS Statistics v.19, IBM Corp., Somers, NY). The linear model used for each dependent variable accounted for the effects of treatment (T), day (d) and Txd interaction. Effects were considered significant at $P \leq 0.05$. When significant differences were detected, differences among means were studied using the LSD comparison test. An ANOVA analysis was used to establish differences in OTUS due to the treatments using R software.

Results

Ruminal fermentation

Methane produced in batch cultures after 24 h of incubation (Table 2) was decreased ($P \leq 0.005$) by PTS (48 %) and BCM (94 %) compared with control. The pH values in fermenters were not affected ($P = 0.308$) by the treatment, although tended to decrease as incubation time progressed ($P = 0.072$). Within treatment total VFA and individual VFA molar proportions differed ($P \leq 0.044$) for days 0 and 4 and then remained unchanged from the day 4 onward. Total VFA concentration decreased ($P < 0.001$) only with BCM addition from day 4 onward compared with control. Acetate molar proportion decreased ($P \leq 0.038$) from day 4 with all the treatments compared with control. Propionate molar proportion increased ($P \leq 0.044$) with all the treatments on day 4, although from day 4 remained higher ($P < 0.001$)

only for BCM treatment. *Iso*-butyrate molar proportion was not affected ($P = 0.222$) by any of the treatments. Molar proportion of butyrate was higher ($P \leq 0.034$) with BCM compared with control and the other two treatments from day 4 onward. Valerate and *Iso*-valerate molar proportions were higher ($P < 0.001$) from day 4 and day 8 onward, respectively, with BCM compared with control and all the treatments. Acetate to propionate ratio decreased ($P < 0.001$) from day 4 onward with BCM compared with other treatments and control. Only on day 4 the ratio was higher ($P = 0.036$) for PTS and on days 4 and 12 it was lower ($P \leq 0.040$) for DDS compared to the control.

The xylanase and amylase activities (Table 3) did not change from day 4 to 12 of incubation ($P > 0.050$). Xylanase activity tended to be affected by PTS treatment ($P = 0.051$) as compared to the control and BCM and DDS. Amylase activity increased ($P < 0.022$) on days 4 and 12 with BCM compared to the control and BCM and DDS.

Microbial community

The effects of the studied additives during 12 days of treatment on the numbers of bacteria, archaea and protozoa are shown in table 4. The abundance of the total bacterial population increased ($P \leq 0.040$) for all treatments on days 4 and 12, with a further increase by BCM treatment as compared with the control. The number of archaeal mcrA gene copies was lower ($P \leq 0.035$) with BCM and PTS from day 4 and from day 4 to 8, respectively, compared with control. No effect ($P = 0.547$) on the numbers of protozoa was observed with any treatment. However, a reduction ($P < 0.001$) of protozoa numbers was observed for all the treatments at day 4 compared to 0. The treatment x day interaction was only significant ($P < 0.001$) for the archaeal gene copy numbers.

The Roche/454 pyrosequencing analysis exhibited 21,530 and 36,183 input sequence reads of bacteria and archaea, respectively. After the removal the low-quality reads, 18,182 bacterial

and 29,404 archaeal reads were used for the analysis.

Alpha diversity based on Shannon diversity and richness of observed species measures showed similar values for bacterial diversity within the treatments, ranging from 8.1, 8.4, 8.5 and 8.7 for BCM, Control, DDS and PTS respectively on Shannon diversity and from 261, 285, 286 and 295 for BCM, Control, DDS and PTS respectively on richness of observed species. Similarly for Archaeal diversity, little variation in the observed sample diversity was observed within treatments with 4.1, 4, 4.1 and 4.1 for BCM, Control, DDS and PTS respectively on Shannon diversity and from 33, 37, 38 and 41 for BCM, Control, DDS and PTS respectively on richness of observed species.

Between treatments diversity as measured with beta diversity analysis for the comparison of the bacterial microbiome structures, found that the BCM treatment contributed the most variance to the Principal Coordinate Analysis (PCoA) (Figure 1a). Although a smaller percentage of variance was also explained in separating the PTS treated samples from the control and DDS samples. Likewise for the archaeal beta diversity, the BCM sample explained the most variance in the data (Figure 1b). However the PTS sample were also clearly separated from the control and DDS samples along the first axis of variance.

Analysis of the Bacteria family level to its specific responses to the inhibitors (based on an average of 4469 sequences per treatment) showed a substantial shift in the relative abundance of some families in fermenters treated with BCM compared to the other three treatments (Fig. 2). For BCM treatment a reduction in *Anaeroplasmataceae* (2.6%) and *Ruminococcaceae* (6.1%) compared with an average of 5.4% and 10.2%, respectively the other treatments. The BCM treatment resulted in greater abundances of *Prevotellaceaea* (15.1%) and *Streptococcaceae* (3.83%) families and of an unclassified family belonging to the order *Bacteriodales* (17.1%) in comparison to the other treatments (9.1%, 1.3% and 12.3%, respectively). Treatment with PTS resulted in higher relative abundance of the

294 *Spirochaetaceae* (5.9%) family compared to the other treatments (2.63%).

295 Specific OTU's that were significantly associated with an increase in abundance due to
296 treatment with BCM ($P \leq 0.05$) were classified to *Butyrivibrio* and *Prevotella* genus, while
297 those decreasing with BCM treatment were classified as *Ruminococcus* genus
298 (Supplementary figure 1). The PTS treatment increased ($P \leq 0.05$) the abundance of some
299 OTUs classified as *Ruminococcus* and *Prevotella* genus. The DDS only affected ($P < 0.001$)
300 one OTU classified as *Clostridium* genus.

301 Analysis of the methanogen genus level to its specific responses to the inhibitors (based on an
302 average of 7351 sequences per treatment) (Fig. 3) revealed higher relative abundance of
303 *Methanobrevibacter* genus in samples from fermenters treated with PTS (79.2%) and BCM
304 (73.7%) than for control (60.6%) and DDS (48.7%) treatments. The relative abundance of
305 archaea belonging to the *Methanosphaera* genus was higher for BCM (25.3%) than for the
306 other three treatments (6.5%). However, the relative abundance of *Methanomicrobium* genus,
307 from the *Methanomicrobiaceae* family was higher for control (32.9%) and DDS (41.4%) than
308 for PTS (11.7%) and BCM (0.3%).

309 In relation to the archaeal community, specific OTU's that were significantly associated with
310 an increase in abundance due to treatment with BCM and PTS ($P \leq 0.05$) were associated
311 (Supplementary figure 2) to *Methanosphaera* and *Methanobrevibacter* genus were increased,
312 while those decreasing with these treatments were classified as *Methanomicrobium* genus.

313

314 ## Discussion

315 In this study the compounds were provided together with the diet twice a day to mimic as
316 close as possible the conditions *in vivo*. Thus, fluctuations in compounds concentration are
317 expected thorough the day but no accumulation along the course of the trial. The selected
318 dosage was made based on previous in vitro trials conducted using batch culture systems

(Martinez-Fernandez *et al*., 2013). Forty ml saliva/h were infused which resulted in 960 ml/d of dilution rate. In contrast to close systems (i.e. batch culture), continuous culture do not have a steady concentration of active compound within the vessel, as it is the case of the rumen.

Ruminal fermentation

Halogenated compounds such as BCM (McAllister & Newbold, 2008), and organosulphur compounds (Patra & Yu, 2012; Mateos *et al*., 2013) such as PTS, have shown antimethanogenic effect in ruminants. In the present work the addition of PTS and BCM decreased methane production by 48% and 98%, respectively, values that are comparable to those reported *in vitro* (Goel *et al*., 2009; Patra & Yu, 2012; Martínez-Fernández *et al*., 2013). On the contrary, DDS did not decrease CH_4 production from fermenters content taken on day 12. We recently reported a reduction in CH_4 production *in vitro* after 24 h treatment with DDS, with equivalent doses as used here (Martínez-Fernández *et al*., 2013). The lack of effect of DDS on methane production using fermenter content taken on day 12 of treatment may suggest an adaptation of the microbial ecosystem over this period to the presence of this compound, which agrees with the results obtained in sheep by Klevenhusen *et al.* (2011), which suggested that some antimicrobial additives may be degraded by rumen microorganisms. The analysis of the microbial community structure in the first days of application of DDS would elucidate the adaptation mechanisms. The antimethanogenic effect of PTS observed in the present work agrees with previous results obtained *in vitro* and *in vivo* in our group (Martínez-Fernández *et al*., 2013). The significance of the treatment by time interaction for some individual VFA molar proportions suggests that the rumen microbial community is altering the fermentation profiles either through functional changes or population shifts in the presence of the studied compounds. Total VFA concentration and

344 profile was only affected by BCM, in contrast to other observations (Goel *et al*., 2009;
345 Mitsumori *et al*., 2012; Abecia *et al*., 2012), which could be due to the higher dose applied
346 here (136 μM) as compared to Goel *et al.* (2009) and Abecia *et al*. (2012) (10 and 23 μM,
347 respectively). Busquet *et al.* (2005) reported no effect of DDS and garlic oil after 9 days of
348 incubation in continuous-culture fermenters on total VFA and modifications of VFA profile
349 with decreased acetate and increased butyrate molar proportions. In the present work
350 acetate:propionate ratio decreased with BCM from day 4 to 12, in agreement with results
351 obtained in previous experiments using the same compound (Mitsumori *et al*., 2012; Abecia
352 *et al*., 2012). This reduction has been considered (McAllister & Newbold, 2008) a common
353 feature for antimethanogenic compounds, as a result of a redirection of hydrogen from
354 methane to propionic metabolic pathways, although the decrease in methane production not
355 necessarily always result in an increase of the propionate production. In this line, some
356 studies (Mitsumori *et al*., 2012; Martínez-Fernández *et al.*, 2014) have reported that most of
357 the hydrogen produced in excess in the rumen when methanogenesis is inhibited is expelled
358 by the animal, which it is very likely to have occurred in our experiment. The potential
359 increase in propionate would result in more energy available for the animal (Abecia *et al.*,
360 2012), athough this effect might depend on the shifts caused in the bacterial populations as
361 discussed below.
362 The treatment with PTS tended to decrease xylanase activity, while BCM and DDS did not
363 show such effect. Xylanase degrades the linear polysaccharide beta-1,4-xylan into xylose,
364 thus breaking down hemicellulose, one of the major components of plant cell walls in
365 forages. The impact that this could have on plant fibre degradation in the rumen and on
366 intakes by animals subjected to high feeding levels deserves further attention. On the other
367 hand, only BCM treatment increased amylolytic activity on days 4 and 12. Other authors
368 (Hristov *et al*., 2003) reported decreased amylolytic activity by several bioactive agents using

bovine rumen fluid, in contrast to our results. These differences could be due to the different activity, chemical structure or mechanism of action of the compounds used in both studies. Janssen (2010) described that cellulolytic microbes produce more acetate and H_2, while amylolytic microbes produce less H_2 and more propionate, which explains why more CH_4 is formed, per unit of fermented matter, from forage based diets as compared to those including concentrate. That could explain the different mechanisms of action of PTS and BCM, so BCM could affect competitors of amylolytic microorganisms, thus increases amylolytic activity and produces less H_2, while PTS could affect other microbial groups as discussed in following sections.

Microbial community analyses

In agreement with the methane reduction observed with the addition of PTS and BCM, the abundance of methanogenic archaea was decreased on days 4 and 8 (and on day 12 only with BCM). Goel *et al.* (2009) reported a sharp decrease in biomass of methanogenic archaea as a result of adding BCM in batch and CCF, in agreement with our results. On the contrary Abecia *et al.* (2012) reported no changes in the concentration of methanogenic archaea in goats treated with BCM for 60 days. This disagreement could be due to the different duration of the treatments (Williams *et al.*, 2009) and the inherent differences between *in vivo* and *in vitro* conditions (Soto *et al.*, 2012; 2013). Some works reported that adding garlic compounds does not induce changes in the abundance of methanogenic archaea (Ohene-Adjei *et al.*, 2008; Kongmun *et al.*, 2011) while others reported decreased archaeal population size (Patra & Yu, 2012). The variability in concentration of active compounds in plant extracts generates confusion because the effects can be contradictory, according to the content of the active component in the extract and the dose used (Patra & Saxena, 2009). Therefore, it seems necessary either to report concentrations and active compounds in plant extracts or to use

pure products to unequivocally define activities, doses, and mechanisms of action.

The taxonomic assignment of bacteria revealed that the most abundant families were *Lachnospiraceae, Ruminococcaceae*, *Prevotellaceae* and unclassified *Bacteroidales* in accordance with other studies (Kong *et al*., 2010; Zened *et al*., 2012). The treatment with BCM increased the relative abundance of *Prevotella* and decreased those belonging to *Ruminococcus,* which is in the line of what Mitsumori *et al*. (2012) observed using the same compound. Increased abundance of *Prevotella* promoted by treatment with BCM is likely associated to the increase in branched chain fatty acids, propionate and amylolytic activity observed in that study. The abundance of *Prevotella* has been linked to hydrogen accumulation due to decreased methane production. Likewise, the effect of BCM on *Ruminococcus* is in agreement with Mitsumori *et al*. (2012) that reported a decrease in *R. albus* as a result of treating goats with BCM, due to the high sensitivity to high partial pressure of hydrogen. This decrease might be compensated by greater abundances of other fibrolytic bacteria such as *F. succinogenes* that does not produce H_2 and is not susceptible to H_2 accumulation. This agrees with Kittelmann *et al.* (2014), who reported that two different bacterial community types are linked with the low-methane emission trait in sheep, hypothesizing that lower CH_4 yields are the result of bacterial communities that ferment ingested feed to relatively less hydrogen, which results in less CH_4 being formed. Furthermore, BCM interferes with the cobalamin-dependent pathway to reduce CO_2 to CH_4 and thus decreasing methanogenesis. Some cellulolityc bacteria have been shown to be dependent on vitamin B12 (Scott & Dehority, 1965), which could explain the shift in the distribution of some bacterial groups observed here and the reduction in the abundance of *Ruminococcus* when treating with BCM. With regards to PTS, although a decrease in methane production was observed, it did not induce detectable changes in the bacterial taxonomic distribution, which could be explained by the fact that the reduction in methane

419 was not as dramatic as for BCM (48 and 94 %, respectively for PTS and BCM) and
420 theoretically no major shift in metabolic H_2 transfer occurred. Indeed, this is confirmed by
421 the PCoA plot in which a distinct group including BCM samples was recognized and
422 separated from the rest.
423 The dominant archaea belonged to the orders *Methanobacteriales* and *Methanomicrobiales*,
424 in accordance with previous works (Janssen & Kirs, 2008; Zhou *et al*., 2009; GU *et al*.,
425 2011). Both BCM and PTS increased the relative abundance of *Methanobrevibacter* and
426 decreased that of *Mathenomicrobium* compared with control and DDS. The BCM treatment
427 also increased archaea from *Methanosphaera*. These results are in agreement with previous
428 results obtained in goats treated with BCM for two months (Abecia *et al*., 2014). At least 60
429 genes are involved in methanogenesis and hydrogen transfer. The first five steps of the
430 pathway result in the sequential reduction of CO2 by electrons sourced from H_2 to form N5-
431 methyl-H4MPT (Thauer *et al*., 1993). The methyl group is then transferred to coenzyme M
432 via the action of methyl-H4MPT:CoM-methyltransferase which is encoded by the mtr gene
433 cluster and this is the step inhibited by BCM. This is a multi-subunit enzyme encoded by
434 more than 10 different genes (Attwood & McSweeney, 2008) that include the methyl CoM
435 reductase cluster (Mcr B, D, C, G, A) and the CoM methyltransferase cluster (Mtr E, D, C, B,
436 A, G, H, X) and a set of genes whose function is currently not known. Within the
437 hydrogenotrophic methanogens, a further 10 genes are conserved (Gao & Gupta, 2007) and
438 include a cluster of genes that encode subunits of [Ni-Fe] hydrogenases (Eha B, C, D, E, F,
439 G) that catalyse the reversible reduction of protons to molecular hydrogen. Although some of
440 the genes are known to be conserved across methanogens, some differ between families
441 (Attwood & McSweeney, 2008). Since our results are consistent with other work using BCM
442 (Abecia *et al*., 2014), we hypothesize that the different sensitivity of Methanobacteriales and
443 Methanomicrobiales is a result of genes differently expressed that make some species more

444 suitable to cope with the new environment.

445 Our results support previous observations by Ohene-Adjei *et al*. (2008) who reported changes
446 in the archaeal banding profile by DGGE as a result of treating sheep with garlic oil. Based
447 on the taxonomy derived from pyrosequencing we hypothesize that the extent in which
448 groups are shifted is directly associated to the extent of methane inhibition. However, the
449 different mechanisms of action of BCM and PTS could also explain the different impact
450 observed. Bromochloromethane directly reacts with reduced vitamin B12 and results in the
451 inhibition of cobamide-dependent methyl group transfer in methanogenesis (Wood *et al*.,
452 1968), while the antimicrobial effect of thiosulfinates (Focke *et al*., 1990; Ruiz *et al.*, 2010) is
453 associated with chemical reaction with thiol groups of various enzymes such as the acetyl-
454 CoA-forming system. Some authors (Busquet *et al.*, 2005; Benchaar & Greathead, 2011)
455 reported the relationship of antimethanogenic effect of organosulphur and the inhibition of
456 HMG-CoA reductase, which play an important role in the synthesis of isoprenoid ethers, the
457 main component of archaeal cell membranes. Therefore, the sensitivity of key archaeal
458 groups to these inhibitory compounds may be explained by the different mechanisms of
459 action exhibited, as discussed above, which would need to be further tested using deep
460 metagenomic and metatranscriptomic sequencing transcription studies combined with pure
461 culture *in vitro* incubations. Kang *et al*., (2013) used a combined RNA and DNA-derived
462 analysis and concluded that less abundant but highly active methanogens may make a greater
463 contribution to total methane formation than their abundance may suggest. Shi et *al.* (2014)
464 have recently observed that in despite of minor changes in the abundance of different
465 methanogens groups in the rumen of sheep that are consistently high or low CH_4 yield
466 phenotypes, the transcription of methanogenesis pathway genes was substantially different.
467 This differential transcription pattern needs to be assessed in the future when effective anti-
468 methanogenesis compounds are applied.

In conclusion, the inhibition of methane production in the rumen by BCM (94%) and PTS (48%) is associated with a shift in the archaeal biodiversity that involves an increase in *Methanosphaera* and a decrease in *Methanomicrobium*. In the case of BCM the effect causes changes in the bacterial population that are clearly reflected in the fermentation profile. The potential negative impact of PTS on fibre degradation deserves further studies. The treatment with DDS over 12 days does not confirm the antimethanogenic effect previously reported.

Acknowledgement

This research was supported by the Science and Innovation Spanish Ministry (Project AGL2008-04707-C02-01). We gratefully acknowledge T. García, E. Jiménez and P. Rufino for their technical assistance and DMC Research Center S.L. for providing PTS. G. Martínez gratefully acknowledges the FPI grant from the Spanish Science Ministry.

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651

651 Table 1. Chemical composition of alfalfa hay and concentrate (g kg^{-1} dry matter) and
652 ingredients (g kg^{-1}) of concentrate.

Item	Alfalfa hay	Concentrate
DM (g kg^{-1} fresh matter)	907	915
OM	875	884
NDF	513	245
ADF	330	118
ADL	99.2	36.3
CP	203	168
Ether Extract	8.1	15.3
GE (MJ Kg^{-1} DM)	18.4	19.5
Ingredients		
Barley		174
Faba beans		233
Maize		174
Sunflower meal		233
Maize gluten meal		116
Rumen-inert fat		70

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655 Table 2. Effects of additives on VFA concentration (mM), profiles (mol 100^{-1} mol) and pH
656 on days 0, 4, 8 and 12 of incubation in continuous-culture fermenters and on CH_4 production
657 (μmol) after 24 hours of incubation in batch culture inoculated with fermenters content after
658 12 days of incubation.

	Day	Treatment				SEM[1]	*P*-value[2]		
Item		Control	BCM	DDS	PTS		T	d	Txd
Total VFA	**0**	30.6^{B}	31.6^{B}	28.8^{B}	28.7^{B}				
	4	76.2^{aA}	62.9^{bA}	74.9^{aA}	73.1^{aA}	11.4	0.015	<0.001	0.013
	8	73.9^{aA}	60.2^{bA}	74.82^{aA}	72.4^{aA}				
	12	77.4^{aA}	65.7^{bA}	78.1^{aA}	78.9^{aA}				
Acetate	**0**	69.0^{A}	68.4^{A}	68.2^{A}	68.4^{A}				
	4	63.7^{aB}	50.0^{cB}	61.4^{bB}	62.8^{abB}	1.8	<0.001	<0.001	<0.001
	8	64.9^{aB}	49.4^{cB}	62.6^{bB}	62.6^{bB}				
	12	64.6^{aB}	49.6^{bB}	61.9^{aB}	62.1^{aB}				
Propionate	**0**	13.8^{B}	14.1^{C}	14.1^{B}	13.9^{B}				
	4	16.0^{bA}	25.8^{aA}	17.6^{bA}	17.1^{bA}	1.5	<0.001	<0.001	<0.001
	8	17.0^{cA}	24.1^{aA}	17.5^{bA}	17.8^{bA}				
	12	17.8^{bA}	23.4^{aB}	18.6^{bA}	18.0^{bA}				
Isobutyrate	**0**	1.88^{A}	1.85^{A}	1.80^{A}	1.83^{A}				
	4	1.48^{B}	1.23^{B}	1.38^{B}	1.43^{B}	0.16	0.163	0.013	0.118
	8	1.33^{B}	1.25^{B}	1.35^{B}	1.23^{B}				
	12	1.30^{B}	1.22^{B}	1.35^{B}	1.35^{B}				
Butyrate	**0**	11.2	11.4^{B}	12.1^{B}	11.9				
	4	13.8^{b}	16.3^{aA}	14.5^{bA}	13.9^{b}	0.7	0.002	<0.001	<0.001
	8	12.2^{b}	15.4^{aA}	13.3^{bAB}	13.8^{ab}				
	12	11.7^{c}	15.6^{aA}	12.9^{bcAB}	13.7^{b}				
Isovalerate	**0**	2.93	3.03	2.70	2.83				
	4	2.78	2.73	2.75	2.50	0.39	<0.001	0.405	<0.001
	8	2.38^{b}	5.38^{a}	2.68^{b}	2.20^{b}				
	12	2.38^{b}	5.90^{a}	2.58^{b}	2.33^{b}				
Valerate	**0**	1.28^{B}	1.28^{B}	1.25^{C}	1.20^{B}				
	4	2.20^{bA}	4.00^{aA}	2.38^{bB}	2.35^{bA}	0.32	<0.001	<0.001	<0.001
	8	2.20^{cA}	4.43^{aA}	2.50^{bAB}	2.38^{bcA}				
	12	2.33^{bA}	4.38^{aA}	2.68^{bA}	2.55^{bA}				
Acetate:Propionate	**0**	5.02^{A}	4.88^{A}	4.90^{A}	4.97^{A}				
	4	3.98^{aB}	1.94^{dB}	3.48^{cB}	3.67^{bB}	0.46	<0.001	<0.001	<0.001
	8	3.85^{aB}	2.06^{bB}	3.57^{aB}	3.52^{aB}				
	12	3.67^{aB}	2.13^{cB}	3.34^{bB}	3.46^{abB}				
pH	**0**	6.55^{A}	6.58	6.55^{A}	6.58				
	4	6.39^{AB}	6.45	6.43^{AB}	6.39	0.22	0.138	0.033	0.668
	8	6.31^{B}	6.44	6.41^{AB}	6.40				
	12	6.36^{AB}	6.44	6.38^{B}	6.40				
Methane	**13**	249^{a}	14^{c}	248^{a}	129^{b}	13	<0.001	n.d.	n.d.

659

660 Treatment: control (without additive), DDS (diallyl disulfide), PTS (propyl propane
661 thiosulfinate) and BCM (Bromochloromethane).

662 [1]SEM: Standard error of the mean.

663 [2] T: treatment effect; d: day effect; Txd: Treatment x day interaction.

664 [a-c] within a row treatment means without a common superscript differ, $P < 0.05$.

665 [A-B] within a column treatment without a common superscript differ, $P < 0.05$.

666

667

667 Table 3. Effect of the additives on xylanase and amylase activities in CCF content sampled
668 on day 4 and 12 after inoculation.

		Treatment				SEM[1]	*P*-value[2]		
Item	Day	Control	BCM	DDS	PTS		T	d	Txd
Xylanase	**4**	5.72	6.52	5.65	5.14	1.58	0.051	0.458	0.186
	12	6.91	7.10	6.08	5.22				
Amylase	**4**	1.03^{b}	1.33^{a}	1.02^{b}	1.04^{b}	0.23	0.022	0.571	0.867
	12	1.10^{b}	1.23^{a}	1.06^{b}	1.08^{b}				

669

670 Treatment: control (without additive), DDS (diallyl disulfide), PTS (propyl propane
671 thiosulfinate) and BCM (Bromochloromethane).

672 [1]SEM: Standard error of the mean.

673 [2] T: treatment effect; d: day effect; Txd: Treatment x day interaction. $^{a\text{-}c}$ within a row
674 treatment means without a common superscript differ, $P < 0.05$.

675 Amylase activity is expressed as micromoles of glucose released from soluble starch by 1 mL
676 of ruminal fluid in 1 min at 39ºC and pH=6.5. Xylanase activity is expressed as micromoles
677 of xylose liberated from oat beachwood xylan by 1 mL of ruminal fluid in 1 min at 39ºC and
678 pH=6.5.

679

679 Table 4. Effects of the additives on the concentration (log copy gene numbers g^{-1} fresh
680 matter) of total bacteria (16S rRNA), protozoa (18S rRNA) and methanogenic archaea
681 (mcrA gene) in fermenters after 0, 4, 8 and 12 days of incubation.

		Treatment				SEM[1]	*P*-value[2]		
Item	Day	Control	BCM	DDS	PTS		T	d	Txd
Methanogens	**0**	8.72	8.63^{A}	8.69^{AB}	8.68^{A}	0.10	<0.001	0.027	<0.001
	4	8.62^{a}	7.91^{bB}	8.17^{abB}	7.96^{abAB}				
	8	8.69^{a}	7.19^{cC}	8.37^{bAB}	8.26^{bB}				
	12	8.81^{a}	6.90^{bC}	8.71^{aA}	8.26^{aAB}				
Protozoa	**0**	8.11^{A}	7.96^{A}	8.14^{A}	8.05^{A}	0.51	0.551	<0.001	0.033
	4	7.23^{B}	7.13^{B}	7.28^{B}	6.96^{B}				
	8	6.93^{B}	7.02^{B}	6.48^{C}	6.74^{B}				
	12	6.79^{B}	6.99^{B}	6.63^{BC}	6.92^{B}				
Bacteria	**0**	9.86^{A}	9.82^{A}	9.88^{A}	9.79^{A}	0.09	0.010	0.002	0.099
	4	9.49^{bB}	9.76^{aA}	9.58^{abB}	9.54^{bABC}				
	8	9.50^{B}	9.56^{B}	9.45^{B}	9.44^{C}				
	12	9.51^{B}	9.74^{A}	9.53^{B}	9.68^{AB}				

682 Treatment: control (without additive), DDS (diallyl disulfide), PTS (propyl propane
683 thiosulfinate) and BCM (Bromochloromethane).

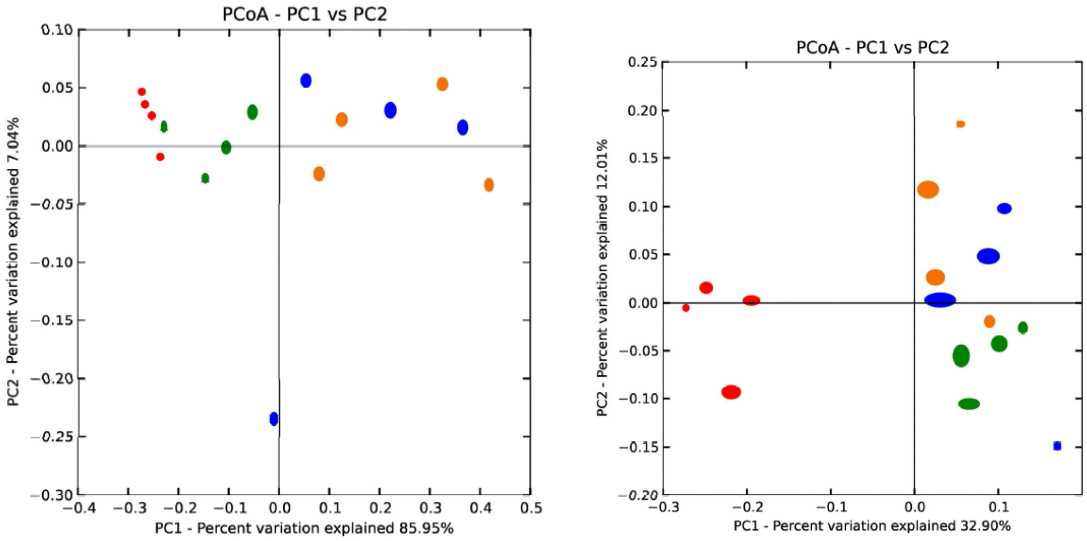
684 [1]SEM: Standard error of the mean.

685 [2] T: treatment effect; d: day effect; Txt: Treatment x day interaction.

686 [a-c] within a row treatment means without a common superscript differ, $P < 0.05$.

687 [A-B] within a column treatment without a common superscript differ, $P < 0.05$.

688
689
690



(A) (B)

Figure 1. Principal coordinate analysis showing the relationships of weighted bacterial (a) and archaeal (b) communities with jackknife support of the fermenters content treated with BCM (Red), PTS (Green), DDS (Orange) and without treatment (Blue) after 12 days of incubation. Size of spots represent robustness of principal coordinate analysis based on jackknife for 1000 subset resamplings.

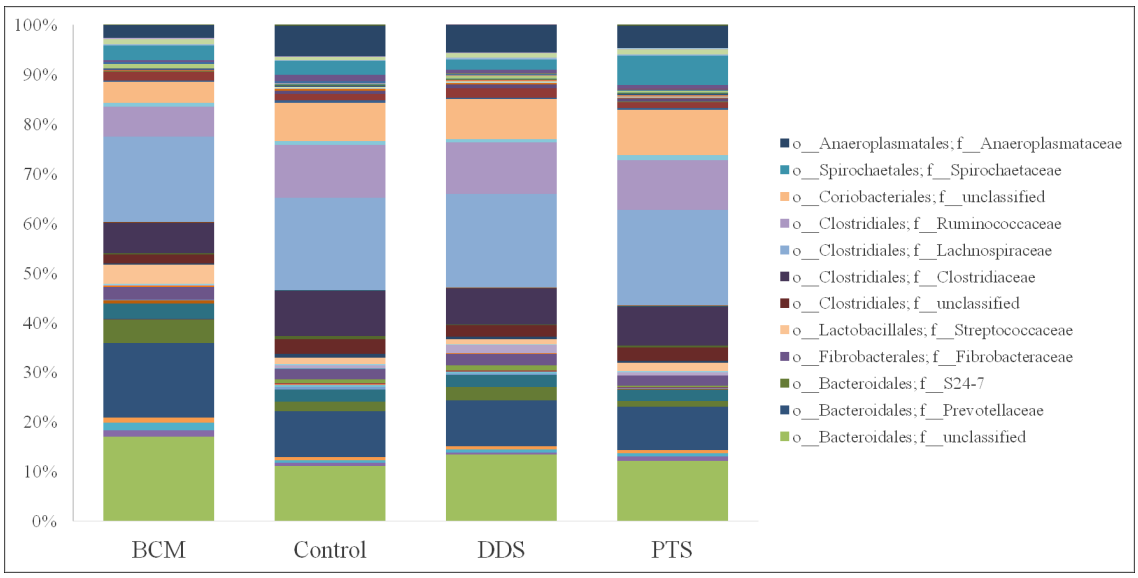


Figure 2. Bacterial taxonomic composition of the fermenters content without treatment (Control), or treated with BCM, DDS and PTS after 12 days of incubation at family level. Sequences were classified using BLAST with a 97% similarity level. Further information regarding the complete family level taxonomic classification is available in supplementary table 1.

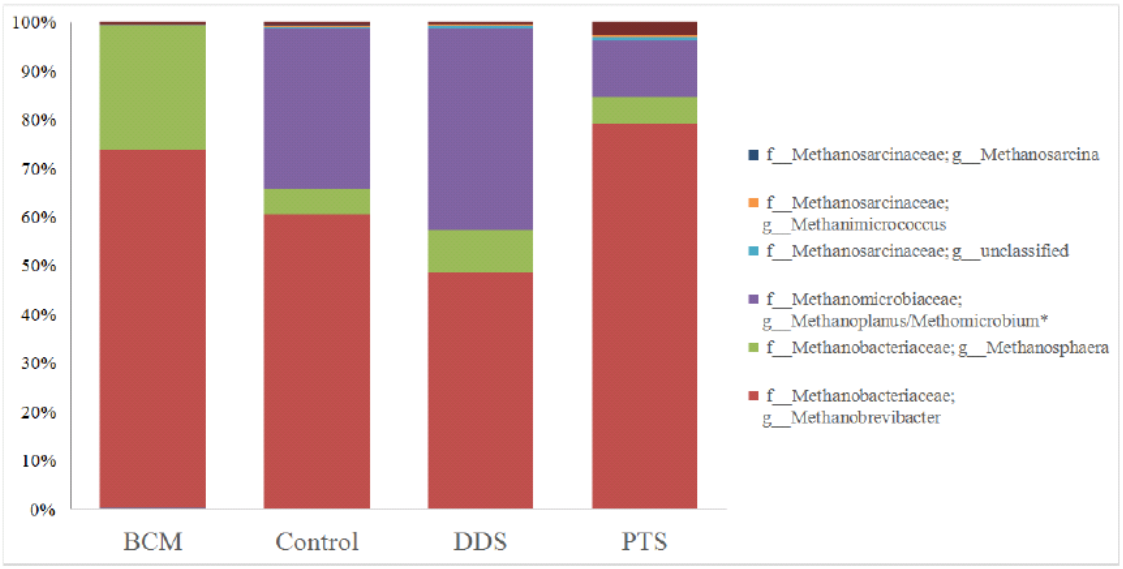


Figure 3. Archaeal taxonomic composition of the fermenters content without treatment (Control), or treated with BCM, DDS and PTS after 12 days of incubation. Sequences were classified using BLAST with a 97% similarity level. Further information regarding the complete family level taxonomic classification is available in supplementary table 2. * Conflict of taxonomy for GreenGenes database. They were most associated to *Methanomicrobium mobile*.